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Novel surface protein (HBsAg) variant of the hepatitis B virus

The invention relates to sequences of a novel variant of the hepatitis B surface antigen (HBsAg) and to methods for detecting this genomic and protein variant as well as antibodies in patient samples which are directed against it.

The novel sequences lead to 11 amino acid substitutions, which have not yet been disclosed in the prior art, in the hepatitis B surface antigen (HBsAg), i.e. in amino acid positions 96 to 136 of the amino acid sequence of the surface antigen, with 10 substitutions being located in the region of the a determinant (aa 101 to aa 180).

The invention also relates to immunochemical detection methods for simultaneously detecting this novel HBV variant together with known variants/subtypes, as well as to the use of the novel sequences in combination with known sequences for simultaneously detecting HBV-specific antibodies. The antigen or antibody determination can in each case be carried out in a test assay which differentiates or does not differentiate.

Finally, the invention also relates to the detection of the corresponding nucleic acids with the aid of nucleic acid tests (e.g. polymerase chain reaction, PCR) using suitable primers, as well as to the use of the novel amino acid sequences for producing vaccines.

As is known, the hepatitis B virus is the agent responsible for a large number of disease courses, ranging from mild inapparent infections through to liver inflammations which are caused by viral infections (viral hepatites), which are chronically active and which take a fulminating course.

With an estimated 400 million persons being affected, chronic infection with HBV constitutes a global health problem (Lee, N. Engl. J. Med. 337; 1733-1745 (1997)).

Active immunization (stimulating the antibody response by administering antigen) and passive immunization (produced by injecting preformed antibodies) are regarded as being the most suitable prophylaxis for the HBV infection which can frequently be encountered world-wide.

HBV belongs to the Hepadna viruses and constitutes a virus particle having a diameter of 42 nm which consists of a core and an envelope. The genome of the virus is a double-stranded, circular DNA sequence of about 3200 nucleotides which encode at least six different viral genes (Tiollais et al., Nature 317: 489-495 (1985)). Four open reading frames are available for forming the viral protein.

The S gene contains the information for the HBV surface antigen (HBsAg), which is also termed small protein (S). In addition, there are also larger forms which are designated large protein (L) and middle protein (M).

All three proteins possess in common the S-HBsAg sequence comprising 226 amino acids (Gerlich et al., Viral Hepatitis and Liver Disease, Hollinger et al., William-Wilkens, Baltimore, MD, pages 121-134 (1991)).

The protein regions upstream of the small HBs are also termed pre-S1 and pre-S2, comprise 108 and 55 amino acids, respectively, and are both present in the L protein (389 amino acids), while the M protein only comprises pre-S2 together with S antigen (281 amino acids). The pre-S proteins exhibit different degrees of glycosylation and carry the receptors for recognizing the liver cells. Unless otherwise indicated, the amino

acid positions in this application refer to the S-antigen (226 aa) without pre-S1 region and without pre-S2 region.

The C gene carries the information for the nucleocapsid protein hepatitis B core antigen (HBcAg). The translation of this protein can already start in the pre-C region and leads to the formation of hepatitis B e antigen (HBeAg). The folding and immunogenicity of HBeAg differs from that of HBcAg. In contrast to HBcAg, HBeAg occurs in free form in serum and, in connection with positive detection, is regarded as an indicator of the formation of HBcAg and consequently of the formation of infectious viral particles.

The reverse transcription DNA polymerase which is present in the virus particle is encoded by the P gene, and the possibility is debated of the transactivator X gene having a causative role in the development of HBV-associated primary liver cell carcinomas.

The viral replication cycle of HBV includes an intracellular pregenomic RNA which is reverse transcribed, in the viral nucleocapsid, into the DNA. Since the reverse transcriptase DNA polymerase which is intrinsic to the HBV does not possess any proof-reading capability, incorrect nucleotides are incorporated at a relatively high frequency. As a consequence, HBV exhibits a mutation rate which, at approx. 1 nucleotide/10 000 bases/infection year, corresponds to about 10 times the rate exhibited by other DNA viruses (Blum, Digestion 56: 85-95 (1995); Okamoto et al., Jpn. J. Exp. Med. 57: 231-236 (1987)).
In addition, deletions and insertions also occur quite frequently (Carman et al., Lancet 341: 349-353 (1993)).

The resulting variability of HBV is manifested, inter alia, in the occurrence of 9 serologically defined

subtypes (Courouce et al., Bibliotheca Haematologica 42: 1 (1976) and a total of at least 6 different genotypes, which are designated A to F (Fig. 1) and are dispersed geographically. (Norder et al., J. Gen. Virol. 73: 3141-3145 (1992), Norder et al., Virology 198: 489-503 (1994)).
In addition, a number of mutants in which 1 amino acid or more has/have been substituted, or is/are missing or supernumerary, have been described.

Aside from mutations which take place naturally (Cooreman et al., Hepatology 30: 1287-1292 (1999)), administering HBV immunoglobulins and/or an antiviral therapy (e.g. using lamivudine) can exert a selection pressure which leads to an increase in the occurrence of what are termed escape mutants and can markedly increase the probability of the appearance of HBV mutants (Terrault et al., Hepatology 28: 555-561 (1998); Tillmann et al., Hepatology 30: 244-256 (1999); Hunt et al., Hepatology 31: 1037-1044 (2000).

Not all HBV mutations result in replication-capable viruses and nonvital and replication-capable viruses frequently coexist, a situation which also limits the precision of the sequencing of isolated DNA or even leads to the failure of PCR, cloning procedures and subsequent sequencing to recognize altered sequences when these latter make up quantitatively less than 10% of the total DNA (Cooreman et al., J. Biomed. Sci. 8: 237-247 (2001).

It is consequently advantageous to isolate mutants, with the subsequent identification and characterization of individual mutants possibly leading to improved vaccines and diagnostic agents.

After an infection with HBV, the immune response is principally directed against what is termed the

a determinant, as a region of the S protein which is common to all hepatitis B viruses, which region is located on the surface of the virus particles (Gerlich et al., see above) and constitutes the most heterogeneous part of the B cell epitopes of the S gene.

According to the present state of knowledge, a total of at least 5 partially overlapping epitopes on the a determinant between amino acid positions 101 and 180 are assumed to be binding sites for antibodies (Figs. 1 and 2), as has been demonstrated by using monoclonal antibodies (Peterson et al., J. Immunol. 132: 920-927 (1984)).

These epitopes are chiefly complex conformational epitopes which are stabilized by several disulfide bridges. Some sequence epitopes, which can be produced using synthetically prepared cyclic peptide structures, are also present.

99% of so-called "protective antibodies", which circulate in serum after a natural infection with HBV, are directed against the very immunogenic a determinant of the HBV (Jilg, Vaccine 16: 65-68 (1998).
The widespread use of immunization with vaccines which have either been isolated from human serum or prepared recombinantly, and the administration of hepatitis B immunoglobulins which contain human HBV-specific antibodies, are based on this fact. Both prophylactic strategies are based on the neutralizing effect which HBs-specific antibodies display after binding to the "a loop epitope" (Carman et al., Hepatology 24: 489-493 (1996), Muller et al., J. Hepatol. 13: 90-96 (1991) and Samuel et al., N. Engl. J. Med. 329: 1842-1847 (1993)).

In a similar manner, the diagnostic agents which are widely used nowadays are based on the binding of

a determinant-specific antibodies to epitopes of the a determinant.
Thus, in the case of the HBsAg determination, using immunochemical determination methods, which is employed world-wide in the field of blood donation, HBV surface antigen which is circulating in the serum of donors is detected using antibodies (of polyclonal or monoclonal origin) which are directed against the a determinant and, if the result is positive, the relevant donated blood is discarded in order to prevent iatrogenic HBV infections due to HBV-contaminated blood. Another application of the HBsAg determination lies in detecting an existing acute HBV infection.

Conversely, a positive result when determining HBs-specific antibodies (anti-HBs) in the blood of test subjects demonstrates that either a natural infection has taken its course or that a vaccination which has been carried out has been successful.

Finally, nucleic acid testing, e.g. by means of the polymerase chain reaction (PCR), is also based on using primers which are specific for the HBV nucleotides.

Due to the central role which the a determinant in active immunization (vaccination with HBV antigen), passive immunization (protection by means of HBV-specific immunoglobulins), detection of the success of a vaccination or of an HBV infection which has taken place (both by means of determining HBsAg-specific antibodies, i.e. anti-HBs) and, finally, safety in the field of blood donation (HBsAg determination and PCR), it is understandable that the appearance of mutants, and also new variants, is followed with great attention in specialist circles.

As a consequence, novel mutants and/or variants which were altered in the a determinant of the HBV, but which

were capable of replication, could be of interest both in connection with prophylaxis and in connection with diagnosis (Brind et al., J. Hepatol. 26: 228-235 (1997), Fischer et al., Transplant Proc. 31: 492-493 (1999), Ghany et al., Hepatology 27: 213-222 (1998), Protzer-Knolle et al., Hepatology 27: 254-263 (1998), Carman et al., Gastroenterology 102: 711-719 (1992) and Coleman et al., WO 02/079217 A1, (2002)).

While there is no sharp differentiation of variants and mutants of HBV, a proposal in this regard is applied widely (Carman, J. Viral Hepat. 4 (suppl. 1): 11-20 (1997). According to this proposal, the designation "variant" should be used for naturally occurring subtypes which appear without any known interference due to selection pressure (antiviral therapy and/or immunoglobulin administration) and exhibit a geographic dispersion pattern.

The characterization and subsequent classification of the subtypes is effected using monoclonal antibodies and is based on a change in the reaction patterns due to one or a few amino acid(s) being substituted. Amino acid positions 122 and 160 of the most widespread HBV sequence: aa 122 and aa 160 = lysine, K, constitute the basis for the classification.

All the serotypes contain the group-specific a determinant while the aa 122 and, in addition, 133 and 134 determine the d or r subtype and aa 160 determines membership of the w or r subtype. On this basis, HBV subtypes can be roughly divided into adr, adw, ayr and ayw, which subtypes can be further differentiated into at least 9 sub-subtypes: ayw1, ayw2, ayw3, ayw4, ayr, adwr2, adw4, adrq+ and adrq- (Swenson et al., J. Virol. Meth. 33: 27-28 (1991), Blitz et al. J. Clin. Microbiol. 36: 648-651, Ashton-Rickardt et al., J. Med. Virol. 29: 204-214 (1989)).

Since this classification is based on serologic reactivity, every typing does not necessarily have to denote variability at the amino acid level, for which reason preference is given to genotyping at the S gene level (Ohba et al., Virus Res. 39: 25-34 (1995).

For reasons not yet known, subtypes appear in particular geographic and ethnic patterns.

According to Carman, the designation mutation should be reserved for variants which arise exclusively under selection pressure such as vaccination or antiviral therapy. Many mutations have already been described, with a number of them giving rise to diagnostically incorrect findings (Carman et al., Lancet 345: 1406-1407); the aa substitutions which are mentioned below are cited as examples of these mutations:

Consensus:	aa Position	Mutant:	
I	110	V	
P	111	T	
T	114	S	
T	116	S	
P	120	T/S	
T	123	A/N	
I/T	126	A/S	
Q	129	H/R	
K/M	133	L	
T	143	M/L	
D	144	H/A/E	
G	145	R/A	
A	157	R	and also

cysteine substitutions in aa positions 107, 124, 137, 147 & 149.

(Coleman, see above; Okamoto et al., Pediatr. Res. 32: 264-268 (1992); Zhang et al., Scand. J. Infect. Dis.

28: 9-15 (1996); Zuckermann et al., Lancet 343: 737-738 (1994)).

Surprisingly, an atypical reaction pattern of hepatitis markers was found in a sample taken from an Egyptian patient (internal number: 118234, withdrawal on 02. Oct. 2002) who had contracted inflammation of the liver.

Aside from the clinical picture involving an increase in the liver values which were typical for such an infection, IgM class hepatitis core antibodies which were detected also indicated an acute HBV infection, without, however, HBsAg being detected when using an approved high-performance HBsAg ELISA.

A PCR which was carried out surprisingly gave a positive result, and sequencing led, entirely unexpectedly, to the nucleotide sequences depicted in Figs. 3 and 4 and to the amino acid sequences depicted in Figs. 5 and 6.

It is clear from these sequences that it is, entirely unexpectedly, not a matter of a point mutation, i.e. the substitution of a few nucleotides, and not a matter, either, of a subtype which might possibly be characterized serologically, since a total of n=11 amino acids in the region from aa 96 to 180 are substituted as compared with the D genotype. In view of the frequency of the amino acid substitutions, it is to be assumed, entirely unexpectedly, that it is a matter of a new wild type or that the mutations are so pronounced that the consequence has more likely to be described as being a new variant, which is designated HDB 11 variant in that which follows.

Analysis of the best agreement of the amino acid sequence of the a determinant with known sequences

points to genotype D (Fig. 1), subtype ayw2 (Fig. 2), from which, however, the new variant surprisingly still differs in 11 aa positions. The 10 substitutions in the region between aa 103 and 136, in accordance with Figs. 1, 5 and 6, constitute the most prominent feature.

Even when account is taken of the possibility that the new HDB 11 variant coexists with a known wild type, the characteristic and novel sequence in the amino acid regions aa 114 to 120, which is to be described as 6 novel amino acid sequences/substitutions (Fig. 1), remains as a surprising feature.

The present invention therefore relates to an oligopeptide or polypeptide which comprises an amino acid sequence which has at least 78% identity with SEQ ID NO: 14. The amino acid sequence shown in SEQ ID NO: 14 corresponds to amino acid positions 93 to 140 of the S antigen of hepatitis B, which antigen has a total length of 226 amino acids. Preferred embodiments relate to an oligopeptide or polypeptide which comprises an amino acid sequence which has at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity with SEQ ID NO: 14.

The invention also relates to an oligopeptide or polypeptide which comprises an amino acid sequence which has at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity with SEQ ID NO: 12. The amino acid sequence shown in SEQ ID NO: 12 corresponds to amino acid positions 43 to 196 of the S antigen of the hepatitis B virus, which antigen has a length of 226 amino acids.

The skilled person is familiar per set with the method for determining the identity between two amino acid sequences, which method can be carried out using customary computer programs. The identity is preferably determined using the "Bestfit" computer program from the Genetics Computer Group (Madison, WI). The parameters are used in the standard (default) settings. Preference is given to using the program version which was current on the priority date of the present application. A high percentage identity means that the two sequences exhibit a high degree of correspondence, identity or equivalence.

The oligopeptide or polypeptide according to the invention can also comprise an amino acid sequence in which from 0 to 10 amino acids are substituted, deleted or inserted as compared with SEQ ID NO: 14. From 0 to 9, from 0 to 8, from 0 to 7, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2 amino acids, or 1 amino acid, can also be substituted, deleted or inserted in the amino acid sequence as compared with SEQ ID NO: 14. Substitutions can also affect the amino acid positions which correspond to positions 96, 103, 114 to 118, 120, 127, 129 and 136 of the S antigen of HBV.

The oligopeptide or polypeptide according to the invention can also comprise an amino acid sequence in which from 0 to 10 amino acids are substituted, deleted or inserted as compared with SEQ ID NO: 12. From 0 to 9, from 0 to 8, from 0 to 7, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2 amino acids, or 1 amino acid, can also be substituted, deleted or inserted in the amino acid sequence as compared with SEQ ID NO: 12.

The oligopeptide or polypeptide according to the invention can also comprise an amino acid sequence in

which from 0 to 9 amino acids are substituted, deleted or inserted as compared with SEQ ID NO: 16. From 0 to 8, from 0 to 7, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2 amino acids, or 1 amino acid, can also be substituted, deleted or inserted in the amino acid sequence as compared with SEQ ID NO: 16.

The oligopeptide or polypeptide of the invention can also comprise an amino acid sequence which is a constituent sequence of SEQ ID NO: 12 containing at least 5 consecutive amino acids of SEQ ID NO: 12, with the constituent sequence at least including one of the positions 54, 61, 72, 73, 74, 75, 76, 78, 85, 87 and 94 of SEQ ID NO: 12. These amino acid positions correspond to positions 96, 103, 114 to 118, 120, 127, 129 and 136 of the S antigen of HBV. The constituent sequence preferably comprises at least 6, more preferably at least 7, most preferably at least 8, consecutive amino acids of the amino sequence shown in SEQ ID NO: 12. In other embodiments, the constituent sequence comprises at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95 or at least 100 consecutive amino acids of the amino acid sequence shown in SEQ ID NO: 12.

The constituent sequence preferably includes two, three, four, five, six, seven, eight, nine, ten, or all eleven, of the positions 54, 61, 72, 73, 74, 75, 76, 78, 85, 87 and 94 of SEQ ID NO: 12.

The polypeptide according to the invention can also comprise a fragment of an HBs antigen of a hepatitis B virus, with the fragment having a length of at least 5 amino acids, the HBs antigen possessing alanine at

position 96, isoleucine at position 103, alanine at position 114, isoleucine at position 115, asparagine at position 116, asparagine at position 117, arginine at position 118, glutamine at position 120, threonine at position 127, histidine at position 129 and/or tyrosine at position 136, and the fragment comprising alanine 96, isoleucine 103, alanine 114, isoleucine 115, asparagine 116, asparagine 117, arginine 118, glutamine 120, threonine 127, histidine 129 and/or tyrosine 136. The oligopeptide or polypeptide can include one, two, three, four, five, six, seven, eight, nine, ten or eleven of these specific amino acid residues.

The shortest length of the oligopeptides or polypeptides according to the invention is 5, preferably 6, more preferably 7, most preferably 8, amino acids. The total length of the oligopeptide or polypeptide is as a rule from 5 to 1000 amino acids, preferably from 6 to 500 amino acids, more preferably from 7 to 300 amino acids, most preferably from 8 to 200 amino acids. The oligopeptides or polypeptides can also contain foreign amino acids which are not encoded by the genome of a hepatitis B virus. Thus, it is possible for amino acids which facilitate coupling solid phases or make possible coupling to labeling substances to be present. It is possible for amino acids which have arisen as a result of the cloning, and which have been concomitantly expressed in association with the recombinant expression, to be present. Finally, the oligopeptide or polypeptide according to the invention can be a fusion protein which, in addition to HBV-derived amino acids, contains a fusion partner, e.g. a tag sequence which facilitates purification, or a protein moiety which increases solubility and/or the yield in association with recombinant expression. Fusion partners of this nature are known per se to the skilled person.

In another embodiment, the oligopeptides or polypeptides do not contain any foreign amino acids which are not encoded by the genome of an HBV. Correspondingly, these oligopeptides or polypeptides are composed of one of the amino acid sequences described above and/or in the claims.

The oligopeptide or polypeptide according to the invention is preferably immunogenic, i.e. it is able to induce an antibody response in a mammalian organism. The oligopeptide or polypeptide customarily contains at least one antigenic determinant or at least one epitope. In a special embodiment, the oligopeptide or polypeptide contains an epitope which is not present in other HBV variants, e.g. in subtype ayw2.

The oligopeptide or polypeptide preferably comprises one of the amino acid sequences SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30.

Another aspect of the invention is an immunogenic peptide or a mixture of immunogenic peptides which contain one or more of the oligopeptides or polypeptides which are described in this application. The immunogenic peptides or the immunogenic mixture can contain the oligopeptide(s) or polypeptide(s) on its/their own or in combination with known HBV immunogens.

The present invention also relates to nucleic acid molecules which are derived from the genome of the novel HBV variant HDB 11 or mutants thereof, in particular nucleic acid molecules which are derived from the gene which encodes HSbAg.

The invention therefore relates, for example, to an oligonucleotide or polynucleotide which comprises a nucleotide sequence which has at least 91% identity with SEQ ID NO: 3. The nucleotide sequence SEQ ID NO: 3 encodes the amino acid sequence SEQ ID NO: 14. Preferred embodiments relate to an oligonucleotide or polynucleotide which comprises a nucleotide sequence which has at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, identity with SEQ ID NO: 3.

The invention also relates to an oligonucleotide or polynucleotide which comprises a nucleotide sequence which has at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, with SEQ ID NO: 1. The nucleotide sequence SEQ ID NO: 1 encodes the amino acid sequence SEQ ID NO: 12.

In this case, identity is defined as the degree of identity between two strands of two DNA segments. The identity is expressed as a percentage, with the number of identical bases in two sequences which are to be compared being divided by the length of the shorter sequence and multiplied by 100 (Smith et al., Adv. Appl. Mathem. 2: 482-489 (1981).

The skilled person is familiar with the method for determining the identity between two amino acid sequences and this method can be carried out using customary computer programs. The identity is preferably determined using the "Bestfit" computer program from the Genetics Computer Group (Madison, WI). The parameters are used in the standard (default) settings. Preference is given to using the program version which was current on the priority date for the present application. A high percentage identity means that the two sequences exhibit a high degree of correspondence,

identity or equivalence.

This assessment can also be applied to amino acid sequences of peptides and proteins (Dayhoff, Atlas of Protein Sequences and Structure, M.O. Dayhoff ed. 5 Suppl. 3: 353-358, Nat. Biom. Res. Found., Washington D.C., USA, Gribskov, Nucl. Acids Res. 14(6): 6745-66763 (1986)).

The invention furthermore relates to an oligonucleotide or polynucleotide which comprises a nucleotide sequence in which from 0 to 13 nucleotides are substituted, deleted or added as compared with SEQ ID NO: 3. From 0 to 12, from 0 to 11, from 0 to 10, from 0 to 9, from 0 to 8, from 0 to 7, from 0 to 6, from 0 to 5, from 0 to 4, from 0 to 3, from 0 to 2 nucleotides, or 1 nucleotide, can also be substituted, deleted or inserted in the nucleotide sequence as compared with SEQ ID NO: 3.

The oligonucleotide or polynucleotide according to the invention can also comprise a nucleotide sequence which is a constituent sequence of SEQ ID NO: 1 containing at least 8 consecutive nucleotides of SEQ ID NO: 1, with the constituent sequence including at least one of the positions 161, 183, 213, 214, 218, 221, 224, 227, 233, 234, 239, 253, 261, 281, 294, 306, 312, 387, 405 and 408 of SEQ ID NO: 1. The constituent sequence preferably comprises at least 9, more preferably at least 10, most preferably at least 12, consecutive nucleotides of the nucleotide sequence shown in SEQ ID NO: 1. In other embodiments, the constituent sequence comprises at least 15, at least 18, at least 20, at least 25, at least 30, at least 35, at least 30, at least 35, at least 40, at least 45, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120, at least 150, at least 175, at least 200, at least 250 or at least 300 consecutive

nucleotides of the nucleotide sequence shown in SEQ ID NO: 1.

The constituent sequence preferably includes two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, 13, 14, 15, 16, 17, 18, 19 or all 20 of the positions 161, 183, 213, 214, 218, 221, 224, 227, 233, 234, 239, 253, 261, 281, 294, 306, 312, 387, 405 and 408 of SEQ ID NO: 1.

In another embodiment, the oligonucleotide or polynucleotide comprises a nucleotide sequence which hybridizes, under stringent conditions and preferably specifically, with a polynucleotide which is complementary to the sequence SEQ ID NO: 1. In yet other embodiments, the oligonucleotide or polynucleotide comprises a nucleotide sequence which hybridizes, under stringent conditions and preferably specifically, with a polynucleotide which is complementary to the sequence SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 and/or SEQ ID NO: 11. The skilled person is familiar per se with methods for determining whether a given oligonucleotide or polynucleotide hybridizes with another polynucleotide. The following conditions constitute a special example of "stringent conditions": a) 16-hour incubation at 42°C in a solution containing 50% formamide, 5×SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate, pH 7.6, 5×Denhardt's solution, 10% dextran sulfate and 20 µg of denatured, sheared salmon sperm DNA/ml; b) subsequent washing in 0.1×SSC at approximately 65°C. Hybridization and washing conditions are known per se to the skilled person and are specified, by way of example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989). A nucleotide sequence hybridizes specifically

with a given polynucleotide when it does not hybridize, or hybridizes much more weakly, with other nucleotide sequences. In the present case, this can mean that the nucleotide sequence does not hybridize, or only hybridizes weakly, with HBsAg-encoding polynucleotides from conventional HBV variants (e.g. genotype D, subtype ayw2).

The invention also relates to an oligonucleotide or polynucleotide which comprises a nucleotide sequence which encodes an oligopeptide or polypeptide according to the invention as described in this application. Another aspect of the invention is an oligonucleotide or polynucleotide which comprises a nucleotide sequence which is complementary to the above-described nucleotide sequences.

The shortest length of the oligonucleotides or polynucleotides according to the invention is 6, preferably 8, more preferably 10, most preferably 12, nucleotides. The total length of the oligonucleotide or polynucleotide is as a rule from 6 to 3000 nucleotides, preferably from 6 to 1500 nucleotides, more preferably from 8 to 900 nucleotides, most preferably from 8 to 600 nucleotides. The oligonucleotides or polynucleotides can also contain nucleotides which are not derived from the genome of a hepatitis B virus. Thus, it is possible for nucleotides which encode particular amino acids which are intended to fulfill desired functions, as described above, to be present. It is possible for nucleotides which have arisen because of the cloning, e.g. in order to insert particular cleavage sites, to be present. Finally, the oligonucleotide or polynucleotide according to the invention can encode a fusion protein which, in addition to HBV-derived amino acids, contains a fusion partner, e.g. a tag sequence which facilitates purification, or a protein moiety which increases

solubility and/or the yield in association with recombinant expression. Fusion partners of this nature, and the DNA encoding them, are known per se to the skilled person.

Preferred oligonucleotides or polynucleotides of the present invention comprise a nucleotide sequence which is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 and SEQ ID NO: 11.

The polynucleotides according to the invention can also be labeled, for example by means of a fluorescent label or a radioactive label. Polynucleotides of this nature can advantageously be employed in a hybridization reaction or a polymerase chain reaction (PCR).

The invention also relates to a vector or a plasmid which contains an oligonucleotide or polynucleotide according to the invention. The plasmid can, for example, be a cloning vector which is used to replicate the nucleic acid in host cells or to make available particular restriction cleavage sites. Expression vectors are vectors which enable the cloned nucleic acid to be expressed in host cells. Various prokaryotic or eukaryotic cells can be host cells. Examples of prokaryotic host cells are bacterial cells such as *E. coli* cells. The expression vectors according to the invention can contain particular control elements such as promoters or sites for binding repression factors. In another embodiment, the expression vectors contain a nucleic acid segment which encodes a part of a fusion protein.

The invention likewise relates to a cell, e.g. a host cell, which harbors a polynucleotide according to the invention, plasmid according to the invention or a

vector according to the invention. The host cells can be cultured under suitable conditions such that transcription of the nucleic acid which is present, and subsequent translation, takes place. The invention also relates to a method for preparing a polypeptide, in which method a polynucleotide, a plasmid or an expression vector of the invention is introduced into host cells and the host cells are cultured under conditions which lead to the polypeptide being expressed. Where appropriate, the polypeptide can subsequently be isolated from the host cells. The polypeptide is preferably prepared in bacteria, most preferably in *E. coli* cells. Suitable means and conditions for the culture are described, for example, in Ausubel et al. (1993) "Current Protocols in Molecular Biology". The expressed polypeptide is isolated using methods which are known per se to the skilled person. Various methods for purifying proteins are described, for example, in Scopes R. (1994) "Protein Purification: Principles and Practice " (3rd edition) Springer Verlag.

However, the polypeptides and peptides of the present invention can also be prepared chemically using known methods such as solid phase synthesis. In the same way, the polynucleotides according to the invention can be prepared using known methods of chemical synthesis. Polynucleotide fragments which have been obtained by means of chemical synthesis can then also be linked enzymically using ligases. The oligonucleotides or polynucleotides according to the invention can also be prepared from known sequences by means of site-directed mutagenesis, with point mutations being inserted at particular positions. Methods of this nature are known per se to the skilled person.

Another aspect of the invention is an antibody which binds to an oligopeptide or polypeptide according to

the invention. These antibodies can be prepared in a known manner, either using an oligopeptide or polypeptide of the invention, e.g. a peptide having one of the sequences SEQ ID NO: 12 to 30, or using a fragment thereof (Harlow and Lane (1988) Antibodies: A Laboratory Manual; Cold Spring Harbor Laboratory). While the antibodies can be polyclonal or monoclonal antibodies, monoclonal antibodies are preferred. The antibodies are preferably specific antibodies which are directed against the HBsAg of the novel HBV variant but which do not recognize HBsAg from other HBV variants, e.g. genotype D subtype ayw2. These antibodies can be obtained by identifying peptides which, on the basis of a comparison of the amino acid sequences of the novel HBsAg and HBsAg from known strains, are specific for the novel HBsAg and using these peptides to prepare the antibodies. It is also possible to prepare a mixture of polyclonal antibodies and to deplete this mixture by incubating it with known HBsAg. In another embodiment, the antibody recognizes known HBsAg variants as well as the novel HBsAg. This makes it possible to detect different variants of HBsAg simultaneously.

The antibody of the invention can bind to an oligopeptide or polypeptide which is composed of an amino acid sequence which is selected from the group consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30. The antibody particularly preferably binds to an oligopeptide or polypeptide which is composed of an amino acid sequence which is selected from the group consisting of SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28,

SEQ ID NO: 29 and SEQ ID NO: 30. In a special embodiment, the antibody does not bind to the a determinants of the known HBV genotypes A, B, C, D, E and F (see Fig. 1). In a special embodiment, the antibody does not bind to the a determinant of HBV genotype D, subtype ayw2.

The invention furthermore relates to an antiidiotypic antibody which represents an amino acid sequence of an oligopeptide or polypeptide according to the invention. Methods for preparing antiidiotypic antibodies are known per se to the skilled person.

The invention also relates to a test kit for detecting hepatitis B viruses, which kit comprises an oligopeptide or polypeptide according to the invention, an oligonucleotide or polynucleotide according to the invention and/or an antibody according to the invention.

The invention also relates to an immunogenic peptide or a mixture of immunogenic peptides which contains one or more oligopeptide(s) or polypeptide(s) according to the invention on its/their own or in combination with known HBV immunogens.

Another aspect of the invention is a method for detecting a hepatitis B antigen, characterized in that (a) a sample is incubated with an antibody according to the invention under conditions which allow the formation of an antigen-antibody complex; and (b) an antigen-antibody complex which contains the antibody is detected.

It is possible to use monoclonal or polyclonal antibodies (or mixtures or fragments thereof or mixtures of fragments) which react with epitopes of the novel HBV variant to determine the a determinant of the

HBV variant according to the invention, in the form of the entire polypeptide sequence or parts thereof, in experimental samples: HBsAg of the HDB 11 variant.

The skilled person is familiar with a large number of determination methods in which immune complexes are formed, or their formation is inhibited, using one or more monoclonal antibody(ies) or polyclonal antibodies (or mixtures thereof or fragments or mixtures of fragments) which is/are specific for the a determinant of the HBV variant.

A special embodiment is the enzyme immunoassay, a possible test principle of which is described below by way of example without, however, restricting the idea of the invention to this principle:

In the very widely used sandwich principle, immobilized antibodies, or fragments thereof, are incubated with the sample under investigation on a suitable support (e.g. microparticles or the surface of wells in a microtitration plate). After excess sample has been removed, HBsAg which is bound to the antibodies is detected by carrying out a further incubation with anti-HBs antibodies (monoclonal or polyclonal or fragments or mixtures of these fragments) which are provided with a probe. The probe employed is frequently an enzyme whose catalytic conversion (after the excess reagent has been removed) of a suitable substrate results in a color reaction which is measured photometrically and whose intensity is proportional to the content of HBsAg which is present in the sample.

Aside from this special embodiment, methods are also known which are homogeneous in nature (i.e. do not required any bound/free separation), which manage entirely without any probe (e.g. agglutination method), which can be evaluated with the naked eye (e.g. radial

immunodiffusion), or which makes use of other probes (e.g. radioactive isotopes or chemiluminescence) or several probes (e.g. the biotin/streptavidin system).

All these embodiments correspond to the prior art, such that, in the case of the present invention, "determining HBsAg of the novel HBV variant" is understood as referring to any methods which are suitable for detecting polypeptide sequences or antigens of the novel HBV variant, irrespective of whether the HBsAg of the novel variant is determined on its own or whether it is determined in combination with HBsAg of known a determinants and/or known mutations in the a region.

It is likewise possible, for economic reasons, to combine an HBsAg determination with a method for detecting another analyte (e.g. HIV antigen or the simultaneous determination of HBV variant HBsAg and specific antibodies directed against it) in one test assay (which is differentiating or nondifferentiating).

The invention also relates to a method for detecting antibodies which are directed against a hepatitis B antigen, characterized in that (a) a sample is incubated with an oligopeptide or polypeptide according to the invention under conditions which allow the formation of an antigen-antibody complex; and (b) the antibody-antigen complex which contains the oligopeptide or polypeptide is detected.

A special embodiment is the enzyme immunoassay, a possible test principle of which is described below by way of example without, however, restricting the idea of the invention to this principle:

In the very widely used sandwich principle, immobilized epitope-carrying polypeptide or protein sequences are

incubated with the sample under investigation on a suitable support (e.g. microparticles or the surface of wells in a microtitration plate). After excess sample has been removed, antibodies which are bound to the epitopes are detected by carrying out a further incubation with epitope-carrying polypeptide or protein sequences which are provided with a probe. The probe employed is frequently an enzyme whose catalytic conversion (after the excess reagent has been removed) of a suitable substrate results in a color reaction which is measured photometrically and whose intensity is proportional to the content of antibody which is present in the sample.

Aside from this special embodiment, methods are also known which are homogeneous in nature (i.e. not require any bound/free separation), which manage entirely without a probe (e.g. agglutination method), which can be evaluated with the naked eye (e.g. radial immunodiffusion) or which make use of other probes (e.g. radioactive isotopes or chemiluminescence) or several probes (e.g. the biotin/streptavidin system).

It is likewise possible for the polypeptide structures of the HBV variant to be represented by antiidiotypic antibodies or, by selecting a suitable test principle, for variant-specific monoclonal or polyclonal antibodies to be used for determining anti-HBs antibodies (in a competitive test format). It is likewise known that, by selecting the test principle, it is also possible to differentiate the immunoglobulin classes (e.g. by means of the "indirect" method using a second class-specific antibody (e.g. IgM- or IgG-specific) possessing any probe or with the aid of what is termed the anti-μ principle (IgM-specific). The methods and materials (incl. probe and polypeptide sequences) naturally have to be adapted to the given aim.

All these embodiments correspond to the prior art, such that, in the case of the present invention, "determining antibodies which are specific for the a determinant of the novel HBD 11 variant" is understood as referring to any methods which are suitable for detecting immunoglobulins and/or immunoglobulin classes directed against the novel HBV variant, irrespective of whether the antibody directed against the novel variant is sought on its own or in combination with antibodies directed against known a determinants and/or known mutations in the a region.

In another method, it is possible to detect a hepatitis B nucleic acid. This method is characterized in that (a) a sample is incubated with an oligonucleotide or polynucleotide according to the invention under conditions which allow the selective hybridization of the oligonucleotide or polynucleotide with a hepatitis B nucleic acid in the sample; and (b) it is determined whether polynucleotide duplexes which comprise the oligonucleotide or polynucleotide have been formed.

The hepatitis B nucleic acid can also be detected by (a) incubating a sample with at least one oligonucleotide or polynucleotide according to the invention under conditions which allow the selective hybridization of the oligonucleotide or polynucleotide with a hepatitis B nucleic acid in the sample; (b) carrying out a polymerase chain reaction; and (c) determining whether a nucleic acid has been amplified.

The invention also relates to the use of an oligonucleotide or polynucleotide according to the invention as a primer and/or as a probe. The present nucleotide sequences can be used for preparing primers and/or gene probes, for which reason kits which

comprise primers and/or probes for detecting HBV variant-specific nucleic acid, either on its own or in combination with known HBV nucleotide sequences, in samples under investigation are likewise part of the subject matter of the invention.

On the basis of the present nucleotide sequences, it is possible to develop primers which can be used in the polymerase chain reaction (PCR). PCR is a method for amplifying a desired nucleotide sequence of a nucleic acid or of a nucleic acid mixture. In this method, the primers are in each case extended specifically by a polymerase using the desired nucleic acid as the reading frame. Following dissociation from the original strand, new primers are hybridized and once again extended by the polymerase. By repetition of these cycles, the sought-after target sequence molecules are enriched.

With reference to nucleic acid tests (NATs), it is possible to use nucleotide sequences of the present invention to prepare DNA oligomers of 6-8 nucleotides or more which are suitable for use as hybridization probes for detecting the viral genome of the HBV variant which is described in individuals who are possibly carrying the virus variant, or, for example in the field of blood donation, for screening stored blood for the presence of the variant genome, either selectively or in combination with detecting nucleotide sequences of known HBV variants and/or HBV mutants.

It is likewise possible, on the basis of the nucleotide sequences of the novel HBV variant which have been found, to develop corresponding primers which are specific for the novel variant or which are able to detect both the novel variant and variants which are known in the prior art.

The present invention furthermore relates to an isolated hepatitis B virus which possesses an HBs antigen which comprises an amino acid sequence having at least 91% identity with SEQ ID NO: 12. The HBs antigen of the virus according to the invention preferably comprises the amino acid sequence SEQ ID NO: 12. Finally, the invention also encompasses cultures of tissue cells which are infected with the HBV variant according to the invention, as well as the isolated HBV variant itself. An immunogenic preparation which contains the attenuated or inactivated HDB 11 variant of HBV is also part of the subject matter of the invention.

The invention also relates to the use of an oligonucleotide or polynucleotide according to the invention, or of an oligopeptide or polypeptide according to the invention, for producing a pharmaceutical for treating or preventing an HBV infection. In particular, the oligonucleotides or polynucleotides or oligopeptides or polypeptides according to the invention can be used for producing a vaccine against HBV.

In addition, the invention also includes a vaccine which comprises a polypeptide of the present invention and a customary adjuvant (e.g. Freund's adjuvant, phosphate-buffered saline or the like). A vaccine of this nature can be used to stimulate the formation of antibodies in mammals. Similarly, the invention encompasses a particle which comprises a non-variant-specific amino acid sequence which induces particle formation together with an epitope-containing polypeptide which is specific for the HBV variant according to the invention.

The nucleotide sequences of the invention can also be used for preparing antisense oligonucleotides (where

appropriate for therapeutic purposes).

Further aspects of the present invention are constituted by the following subject- matter items (1) to (21):

(1) An isolated oligonucleotide or polynucleotide having one of the sequences selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 11:

SEQ ID NO:1

127 GGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCAC
TCACCAACCTCCTGTCCTCCAACTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTT
ATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGCTCTTCTGGACTAT
CAAGGTATATTGCCCGTTTGTCCTCTAATTCCAGGATCTGCAATCAACAACAGGGGACAA
TGCAAAACCTGCACGACTACTGCTCACGGAACCTCTATGTATCCCTACTGTTGCTGTACC
AAACCTTCGGACGGAAATTGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAA
TTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTTCCCTTTGTT
CAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGG 588

SEQ ID NO:2

277 TTCTTGTTGGCTCTTCTGGACTATCAAGGTATATTGCCCGTTTGTCCTCTAATTCCA
GGATCTGCAATCAACAACAGGGGACAA 360

SEQ ID NO:3

277 TTCTTGTTGGCTCTTCTGGACTATCAAGGTATATTGCCCGTTTGTCCTCTAATTCCA
GGATCTGCAATCAACAACAGGGGACAATGCAAAACCTGCACGACTACTGCTCACGGA
ACC TCTATGTATCCCTACTGTTGCTGTACC 420

SEQ ID NO:4

301 CAAGGTATATTGCCCGTTTGTCCTCTAATTCCAGGATCTGCAATCAACAACAGG
GGACAATGCAAA 366

SEQ ID NO:5

301 CAAGGTATATTGCCCGTTTGTCCTCTAATTCCAGGATCTGCAATCAACAACAGG
GGACAATGCAAAACCTGCACGACTACTGCTCACGGAACCTCTATGTATCCCTACTGT
TGCTGTACC 420

SEQ ID NO:6

340 GCAATCAACAACAGG 354

SEQ ID NO:7

340 GCAATCAACAACAGGGGACAA 360

SEQ ID NO:8

340 GCAATCAACAACAGGGGACAATGCAAA 366

SEQ ID NO:9

340 GCAATCAACAACAGGGGACAATGCAAAACCTGCACGACTACTGCTCAC 387

SEQ ID NO:10

340 GCAATCAACAACAGGGGACAATGCAAAACCTGCACGACTACTGCTCACGGAACC
TCTATGTATCCCTACTGTTGCTGTACC 420

SEQ ID NO:11

361 TGCAAAACCTGCACGACTACTGCTCACGGAACCTCTATGTATCCCTACTGTTGC
TGT ACC 420

(2) An oligonucleotide or polynucleotide according to (1) which is in each case at least 65% or 66% or 67% or 68% or 69% or 70% or 71% or 72% or 73% or 74% or 75% or 76% or 77% or 78% or 79% or 80% or 81% or 82% or 83% or 84% or 85% or 86% or 87% or 88% or 89% or 90% or 91% or 92% or 93% or 94% or 95% or 99% or 97% or 98% or 99% identical with one of the sequences selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 11.

(3) An oligonucleotide or polynucleotide according to (1) or (2) which hybridizes, under stringent conditions, with an oligonucleotide or polynucleotide which has a sequence which is complementary to one of the sequences selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 11.

(4) An isolated oligonucleotide or polynucleotide which encodes HBs antigen of the hepatitis B virus and contains an oligonucleotide or polynucleotide according to (1), (2) or (3).

(5) A fragment of an oligonucleotide or polynucleotide which encodes HBs antigen of the heptatitis B virus, characterized in that the fragment contains an oligopeptide or polypeptide according to (1), (2) or (3).

(6) An isolated oligonucleotide or polynucleotide which encodes the a determinant of the HBs antigen of the heptatitis B virus and contains an oligonucleotide or polynucleotide according to (1), (2) or (3).

(7) A primer which is specific for an oligonucleotide or polynucleotide according to one of the subject-

matter items (1) to (6).

(8) A vector which contains at least one oligonucleotide or polynucleotide according to one of the subject-matter items (1) to (5).

(9) A host cells which harbors a vector according to (8).

(10) An oligopeptide or polypeptide which is encoded by an oligonucleotide or polynucleotide according to one of the subject-matter items (1) to (5).

(11) An isolated oligopeptide or polypeptide which has an amino acid sequence which is selected from the group consisting of SEQ ID NO: 12 to SEQ ID NO: 30:

SEQ ID NO:12

43 G G T T V C L G Q N S Q S P T S N H
S P T S C P P T C P G Y R W M C L R R F
I I F L F I L L L C L I F L L A L L D Y
Q G I L P V C P L I P G S A I N N R G Q
C K T C T T T A H G T S M Y P Y C C C T
K P S D G N C T C I P I P S S W A F G K
F L W E W A S A R F S W L S L L V P F V
Q W F V G L S P T V W L S V I W 196

SEQ ID NO:13

93 F L L A L L D Y Q G I L P V C P L I P G
S A I N N R G Q 120

SEQ ID NO:14

93 F L L A L L D Y Q G I L P V C P L I P G
S A I N N R G Q C K T C T T T A H G T S
M Y P Y C C C T 140

SEQ ID NO:15

101 Q G I L P V C P L I P G S A I N N R G Q
C K 122

SEQ ID NO:16

101 Q G I L P V C P L I P G S A I N N R G Q
C K T C T T T A H G T S M Y P Y C C C T 140

SEQ ID NO:17

114 A I N N R 118

SEQ ID NO:18

110 I P G S A 114

SEQ ID NO:19

111 P G S A I 115

SEQ ID NO:20

112 G S A I N 116

SEQ ID No.: 21

113 S A I N N 117

SEQ ID NO:22

115 I N N R G 119

SEQ ID NO:23

116 N N R G Q 120

virus, characterized in that the fragment contains an oligopeptide or polypeptide according to one of the subject-matter items (10) to (12).

(15) An isolated polypeptide which encodes the a determinant of the HBs antigen of the hepatitis B virus, characterized in that it contains an oligopeptide or polypeptide according to one of the subject-matter items (10) to (12).

(16) A monoclonal or polyclonal antibody which binds to HBs antigen containing an oligopeptide or polypeptide according to one of the subject-matter items (10) to (15) but which does not bind, or at least binds significantly more weakly, to HBs antigen belonging to a hepatitis B wild-type virus.

(17) An antiidiotypic antibody which represents an amino acid sequence according to one of the subject-matter items (10) to (15).

(18) A test kit for detecting or determining, by means of a hybridization reaction, a nucleic acid which is specific for a variant or mutant of the hepatitis B virus using at least one oligonucleotide or polynucleotide according to one or more of the subject-matter items (1) to (7).

(19) A test kit for immunochemically detecting or immunochemically determining an antigen which is specific for a variant or mutant of the hepatitis B virus using at least one monoclonal or polyclonal antibody according to (16).

(20) A test kit for immunochemically detecting or immunochemically determining an antibody directed against a variant or mutant of the hepatitis B

virus using at least one oligopeptide or polypeptide according to one of the subject-matter items (10) to (15).

(21) An immunogenic peptide or mixture of immunogenic peptides which contains one or more oligopeptides or polypeptides according to one or more of the subject-matter items (3) and (4) on its own or in combination with known HBV immunogens.

The present invention encompasses an isolated nucleotide sequence which is at least 65% identical with SEQ ID NO: 1 or with a fragment of this sequence depicted in Figs. 3 and 4 which hybridizes specifically with the complement of SEQ ID NO: 1 to 11.

In addition, the present invention encompasses an isolated nucleotide sequence which encodes the present variant according to the invention of the a determinant of the hepatitis B surface antigen (HBsAg) in the amino acid positions between aa 101 and 180 or leads to a peptide product whose aa sequence is in at least 65% agreement with the SEQ ID NO: 12 depicted in Figs. 5 and 6 or fragments thereof in accordance with SEQ ID NO: 13 to 30.

The present invention furthermore relates to a vector which comprises one or more of said nucleotide sequences as well as to a host cell which harbors this vector and to a method for preparing a corresponding polypeptide from the a determinant, which method comprises incubating the abovementioned host cell over periods and under conditions which are required for expressing the polypeptide.

The invention also relates to antibodies which react with the a determinant described in SEQ ID NO: 12 to 30, with the binding preferably taking place in the

amino acid region aa 101 to 150. The antibodies can be of polyclonal or monoclonal, animal or human origin.

The invention likewise relates to an isolated HBV variant, with the virus possessing an a determinant which corresponds to the aa sequences at least between position 101 and 120 and/or aa 121 to 140, ideally to both said regions between 101 and 140.

The present invention also relates to an immunogenic mixture for generating polyclonal or monoclonal antibodies, which mixture comprises the described, isolated HBV or one or more of the described polypeptides.

The invention also encompasses a polynucleotide probe which contains an HBV genome sequence which, by substitution of amino acids, leads to a modified a determinant which is identical with the described aa sequence of the novel HBV variant or is in at least 65% correspondence with it.

The invention also relates to kits for detecting polynucleotides of the HBV variant with the aid of said probe as well as to kits for detecting HBsAg of the variant or individual epitopes thereof and to antibodies which are specific for the variant or epitopes thereof, as well as to the methods for detecting polynucleotides, antigen and antibody, comprising an incubation for forming corresponding complexes and detection of these complexes using suitable methods known to the skilled person.

The embodiments of these kits and detection methods can be designed for the specific and sole detection of nucleotides and antigens of the HBV variant, or of antibodies directed against them, or be supplementary, i.e. permit detection of the variant analyte according

to the invention in addition to currently known HBV nucleotides, antigens or antibodies.

In an analogous manner, an immunogenic mixture of polypeptide sequences according to the invention can also be used in combination with known antigens, e.g. for improving the efficacy of the vaccine.

The present invention describes a novel variant of the hepatitis B virus (HBV) which possesses an entirely novel a determinant as a result of amino acid substitutions in the following aa positions of the S-HBsAg sequence. The single-letter code is used for describing the amino acids:

aa of HDB 11	aa position	aa of ayw2/genotype D
I	103	M
A	114	S
I	115	T
N	116	T
N	117	S
R	118	T
Q	120	P
T	127	P
H	129	Q
Y	136	S

In addition, alanine (A) is present in place of valine (V) in position aa 96 of HDB 11:

A	96	V

These aa substitutions can be attributed to corresponding nucleotide substitutions in the corresponding codons.

The present invention relates to an isolated nucleotide sequence which encodes the a determinant of the virus (Fig. 3 and also SEQ ID NO: 1).

The invention also encompasses nucleotides having at least 65% congruence, preferably at least 75% congruence, and particularly preferably having at least 90% congruence, with the nucleotide sequence of the present invention, or fragments thereof, as well as sequences which are complementary thereto.

The invention also encompasses polypeptides which are encoded by above-described nucleotide sequences, in particular those amino acid sequences which determine the a determinant of the HBsAg, and polypeptides which at least exhibit a similarity of 65%, preferably 75%, and even more preferably 95%, to these sequences.

For the description of the present invention, a nucleotide fragment is understood as being a consecutive sequence of at least 8 or 9, preferably 9-15, particularly preferably 15-21, and even very particularly preferably 21-60, nucleotides from the nucleotide sequence of the novel HBV variant, with mixtures of these nucleotide fragments also being encompassed.

A polypeptide fragment is understood as being a sequence of at least 3, preferably 3-5, particularly preferably 5-7, and even very particularly preferably 7-20, amino acids from the a determinant of the novel HBV variant, with mixtures of such polypeptide fragments also being encompassed by this invention.

The present invention also encompasses an isolated nucleotide sequence which can be hybridized and leads to nucleotide sequences which correspond to the nucleotide sequences of the HBsAg of the novel HBV variant or parts of the a determinant of the novel HBV variant, are complementary thereto, or are to be traced back to HDB 11 as a subtype or mutation.

The skilled person is familiar with the fact that, after its isolation using methods in accordance with the prior art, a nucleotide sequence can be introduced into prokaryotic (e.g. E. coli) or eukaryotic host cells (e.g. Chinese hamster ovary cell) or yeast (e.g. S. Cerevisiae) with the aid of a vector or construct (using methods known to the skilled person such as transfection, transformation or electroporation: Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, ed Sambrook et al., Cold Spring Harbor Laboratory Press (1989), with it being possible to use transient or permanent cultures.

Consequently, the present invention encompasses isolated nucleotide sequences of the a determinant of the novel HBV variant, polypeptides which are encoded by these nucleotides, vectors which contain nucleotide sequences of the a determinant of the novel HBV variant, and also the host cell into which a vector is introduced. In addition to using an expression system to prepare polypeptides (recombinantly), the skilled person is familiar with the fact that it is also possible to prepare analogous polypeptide structures synthetically or directly by purification from the virus variant.

It is possible to use the polypeptides or proteins of the novel HBV variant to generate monoclonal and/or polyclonal antibodies which bind immunologically to binding sites (epitopes) of the a determinant of the novel HBV variant. The methods for preparing antibodies are known to the skilled person (e.g. Koehler et al., Nature 256-495 (1975), Mimms et al., Vi. 176: 604-619 (1990).

It is furthermore possible to use the a determinant of the HDB 11 variant according to the invention in the

form of the entire polypeptide sequence or parts thereof, for determining antibodies (anti-HBs antibodies) which are directed against the HBV variant (see above).

The skilled person is familiar with a large number of determination methods in which immune complexes are formed, or their formation is inhibited, using polypeptides from the a determinant of the HBV variant and antibodies of animal or human origin.

Finally, it is possible to use monoclonal or polyclonal antibodies (or mixtures or fragments thereof or mixtures of fragments) which react with epitopes of the novel HBV variant to determine the a determinant of the HBV variant according to the invention in the form of the entire polypeptide sequence, or parts thereof, in samples under investigation: HBsAg of the HDB 11 variant.

The skilled person is familiar with a large number of determination methods in which immune complexes are formed, or their formation is inhibited, using one or more monoclonal antibody(ies) or polyclonal antibodies (or mixtures thereof or fragments or mixtures of fragments) which are specific for the a determinant of the HBV variant.

It is likewise possible to develop corresponding primers on the basis of the nucleotide sequences of the novel HBV variant which have been found.

Finally, the invention also relates to diagnostic reagents as kits which, based on the above-described methods detection of HBV variant-specific antigen (HBsAg) or antibodies directed against it (anti-HBs), either as single determinations or can be combined with each other or with other known HBV antigens or

antibodies which react specifically therewith or else with quite different analytes.

In addition, the present invention is described in the patent claims.

Description of the figures:

Fig. 1 presents an overview of the amino acid sequences of the a determinant of 6 described HBV genotypes in comparison with HDB 11.

Fig. 2 depicts the nucleotide and amino acid sequences of the a determinant, as well as immediately adjacent regions of the HBV genotype D, subtype ayw2.

Fig. 3 shows the nucleotide sequence of the a determinant of the HBV surface antigen for subtype ayw2 of HBV genotype D as compared with the nucleotide sequence of HDB 11.

Fig. 4 summarizes the translation-relevant differences in the nucleotide sequence of HDB 11.

Fig. 5 depicts the nucleotide sequence of HDB 11 in the region of the a determinant, as well as the corresponding amino acid sequence. The a determinant is located between amino acids No. 101 and 180 of the small HBsAg (small, S).

Fig. 6 shows the corresponding polypeptide sequence of the a determinant of HDB 11, which polypeptide sequence is encoded by the nucleotide sequence described in Fig. 5.

The following examples explain the present invention in more detail, without the invention being restricted to the examples which are described.

Example 1: Using enzyme immunoassay, EIA, to determine HBsAg

The enzyme immunoassay Enzygnost® HBsAg 5.0 from Dade Behring GmbH, Marburg, Germany, was used to determine the HBV surface antigen, i.e. HBsAg, in the blood of the Egyptian patient.

It is a high-performance test which is approved in Europe and which was performed in accordance with the instructions in the pack information leaflet.

The underlying test principle is a sandwich test in microtiter plate format:
100 µl of the sample to be investigated are brought into contact, in a one-step method, with 25 µl of conjugate 1 (mouse monoclonal HBsAg-specific antibodies which are covalently labeled with biotin) and immobilized sheep polyclonal HBsAg-specific antibodies. After a 60-minute incubation at 37°C, and after removing excess components by washing the plate wells 4 times, 100 µl of conjugate 2, which consists of streptavidin to which the probe enzyme peroxidase is covalently bonded, are added.
After a 30-minute incubation at 37°C, and after having removed excess components by washing the plate wells 4 times, 75 µl of chromogen buffer/substrate solution are added, with this being followed by a 30-minute incubation at room temperature. The development of the blue tetramethylbenzidine dye is terminated by adding 75 µl of stopping solution (sulfuric acid) and the dye is measured photometrically at 450 nm.

The intensity of the color which develops, as measured by the optical density (O.D.), is directly proportional to the content of HBsAg in the investigated sample, with an O.D. value of less than the threshold value

being assessed as HBsAg-negative. The threshold value is defined as the mean value of the O.D. of the negative control (contained in the test kit) which is tested in parallel, to which a constant quantity of 0.05 O.D. is added.

The detection limits of the batch (# 32874) which was used for the investigation were determined, by means of graphic interpolation and using the internationally accepted standard preparations from the Paul Ehrlich Institute, Langen, Germany, to be 0.012 ng of ad subtype/ml and, respectively, 0.015 ng of ay subtype/ml in parallel with the experimental assays from tests of dilutions of the standard preparations in HBsAg-negative serum.

Analysis of the sample # 118234 (withdrawn on 02. Oct. 2002 and from which the DNA was also isolated) gave results of 0.04 and 0.05 O.D. in 2 independent experiments on two different days, which results are to be interpreted, in accordance with the criteria of the test, as being HBsAg-negative. On the other hand, the positive control (contained in the test kit) which was concomitantly assayed was as positive (validation criteria fulfilled) as the abovementioned ad and ay standard preparations.

Example 2: Isolating the HDB 11 DNA from sample # 118234

The QIA amp® DNA blood mini kit from Qiagen, Hilden, Germany, was used to isolate the DNA from a 200 µl aliquot of the Egyptian sample. In doing this, all the procedural steps were followed as described in the pack information leaflet and the elution was performed in a volume of 50 µl.

Example 3: Polymerase chain reaction, PCR

3.1 HBV primers

The four HBV primers listed below were used:

Primer 1 having the 5'>3' sequence:
GGGTCACCATATTCTTGGGAAC (SEQ ID NO: 31)
Primer 2 having the 5'>3' sequence:
TATACCCAAAGACAAAAGAAAATTGG (SEQ ID NO: 32)
Primer 3 having the 5'>3' sequence:
GACTCGTGGTGGACTTCTCTC (SEQ ID NO: 33)
Primer 4 having the 5'>3' sequence:
TACAGACTTGGCCCCCAATACC (SEQ ID NO: 34)

3.2 PCR amplification

The Perkin Elmer Ampli Taq ® DNA polymerase kit as well as the Thermocycler Gene Amp ® PCR system 9700 from Perkin Elmer Applied Biosystems, USA, were used to carry out a nested PCR amplification of the surface antigen.

The nucleotides were obtained from Amersham Biosciences, UK.

For the first amplification cycle, 5 µl of the isolated DNA were amplified using the abovementioned primers 1 and 2 and the following conditions:

PCR 1 rxn

Primer 1 (10 µM)	1 µl
Primer 2 (10 µM)	1 µl
10-fold conc. buffer (incl. 15 µM Mg2Cl)	5 µl
dNTP mixture (10 µM)	1 µl
dist. Water	36.75 µl
Ampli Taq (5 U/µl)	0.25 µl
per tube	45 µl total volume

plus	5 µl	of isolated DNA
	50 µl	reaction volume

The 50 µl assay was amplified using the above-described thermocycler under the following conditions:

94°C, 1 min./94°C, 28 sec. - 55°C, 28 sec. - 72°C, 38 sec. (35 cycles)/72°C, 5 min./8°C soak.

In the second round of amplification, 5 µl of the first PCR product were further amplified using the HBV primers 3 and 4 and the following conditions:

PCR 2 rxn

Primer 3 (10 µM)	1 µl
Primer 4 (10 µM)	1 µl
10-fold conc. buffer	5 µl
dNTP mixture (10 µM)	1 µl
dist. water	36.75 µl
Ampli Taq (5 U/µl)	0.25 µl
per tube	45 µl total volume
plus	5 µl of PCR product v.rxn
	50 µl reaction volume

This PCR 2 assay was amplified using the above-described thermocycler and employing the following conditions:

94°C, 1 min./94°C, 28 sec. - 55°C, 28 sec. - 72°C, 38 sec. (35 cycles)/72°C, 5 min./8°C soak.

In conclusion, the PCR 2 product was fractionated electrophoretically (1.5% agarose) while including suitable molecular weight markers. The band containing approx. 520 base pairs was excised and isolated using the QIA quick gel extraction kit

from Qiagen, Hilden, Germany.

Example 4: Sequencing HDB 11

The purified PCR product was sequenced by Medigenomix, Martinsried, Germany, with the aid of the ABI 3700 Kapillar system in combination with the ABI BigDye Terminator Chemistry Version 1.1. and the ABI Sequencing Analysis Software Version 3.6. and using the primers 3 and 4 described in Example 3.

Sequencing results

It was shown that, while the nucleotide and amino acid sequences within the sequence region of the HBsAG in the sample exhibited the best congruence with genotype D, subtype ayw2, there were a total of 10 amino acid substitutions in the region of the a determinant (see also Figs. 2 and 5):

HDB 11:		**D, ayw2:**
1.)	Ile (I)	substituted for 103 Met (M)
2.)	Ala (A)	substituted for 114 Ser (S)
3.)	Ile (I)	substituted for 115 Thr (T)
4.)	Asn (N)	substituted for 116 Thr (T)
5.)	Asn (N)	substituted for 117 Ser (S)
6.)	Arg (R)	substituted for 118 Thr (T)
7.)	Gln (Q)	substituted for 120 Pro (P)
8.)	Thr (T)	substituted for 127 Pro (P)
9.)	His (H)	substituted for 129 Gln (Q)
10.)	Tyr (Y)	substituted for 136 Ser (S)

In addition, there is an amino acid substitution at position # 96:

11.)	Ala (A)	substituted for 96 Val (V)

These results were reproduced, with the same sequencing

results, in several independent experiments performed on different primary tubes of the blood sample taken on 02. Oct 2002.

The only exception was that, in the first analysis, the position aa# 122 was read as Arg R while the second analysis indicated that Lys (I) was more likely. The nucleotide profile allows both interpretations, something which, while possibly also suggesting that two hepatitis B viruses of differing subtype (ad and, especially ay) might be coexisting, does not change the amino acid sequence or the conclusion about the existence of a novel variant containing the abovementioned amino acid substitutions. This conclusion is reached, inter alia, from the negative EIA result, since, in view of the very good detection limits of the EIA determination method employed, significant quantities of circulating HBsAg of known structure would have in any case had to have given rise to a positive EIA result.

The amino acid which corresponds to position 122 can therefore either be K or R in the sequences SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30.